

Review

MACROMOLECULAR CROWDING UPON IN-VIVO-LIKE ENZYME-KINETICS: EFFECT OF ENZYME-OBSTACLE SIZE RATIO

*Cristina Balcells¹, Isabel Pastor^{1,2}, Laura Pitulice³,
Claudia Hernández¹, Mireia Via¹, Josep Lluís Garcés⁴,
Sergio Madurga¹, Eudald Vilaseca¹, Adriana Isvoran³,
Marta Cascante⁵, and Francesc Mas^{1,*}*

¹ Department of Physical Chemistry and Research Institute of Theoretical and Computational Chemistry (IQTUB) of Barcelona University, C/ Martí I Franquès, 1, 08028-Barcelona (Spain)

² Small Biosystems Lab, Department of Fundamental Physics, University of Barcelona, Barcelona and CIBER-BBN, Carlos III Health Institute, Madrid (Spain)

³ Department of Biology-Chemistry, West University of Timisoara, Timisoara (Romania)

⁴ Department of Chemistry, University of Lleida, Lleida (Spain)

⁵ Department of Biochemistry and Molecular Biology and Institute of Biomedicine (IBUB) of Barcelona University, Barcelona (Spain)

ABSTRACT

In the present work, the volume exclusion phenomenon, also known as macromolecular crowding, has been applied to the field of enzyme kinetics. It has been approached by adding polymeric obstacles in the media of different enzymatic reactions. The concentration and size of these obstacles have been changed systematically in order to obtain kinetic information about each reaction. Results indicate that the performance of a certain enzyme always depends on the amount of excluded volume. However, only large, oligomeric proteins display an obstacle size-dependent behavior. Besides, crowding can hinder diffusion to the extent of being capable of shifting reaction control from activation to diffusion.

Keywords: Enzyme kinetics, crowding, Dextran, excluded volume effects, enzymatic reaction control

* corresponding author: fmas@ub.edu

1. INTRODUCTION

Physicochemical characterization of biomolecules, both theoretically and experimentally, has been traditionally developed in dilute solution conditions. This scenario, even though easy to study and close to ideal condition, does not resemble the real situation inside cells: the cell cytosol contains macromolecules up to 300-400 g/L, and the space in it is highly structured and compartmented [1].

However, studying biomolecules in their natural environment is still impossible for most biological processes at molecular scale, and it can lead to a dead end: having such a great number of variables that the outcome may be impossible to interpret and comprehend. Thus, all the interrelations between the biological system (e.g. a protein and its substrates) and its environment must be studied separately in a model system.

Macromolecular crowding aims to mimic the high levels of excluded volume existing in the cell and tries to discern how this can affect any physicochemical process occurring inside [2,3]. It is achieved by experimentally modelling the cytosol using a wide variety of neutral, relatively inert and random-coil shaped macromolecules, such as Dextrans, Ficolls or Polyethylene glycol (PEG).

Ultimately, an *in-vivo*-like environment is sought, an *in vitro* environment that truly reconstructs the cell cytosol by all means: obstacles of different sizes all together, confined spaces, filamentous structures similar to the cytoskeleton... Yet, this is still far away since the effect of excluded volume with homogeneously sized, coil-shaped obstacles is yet to be fully understood. Such artificial recreation of the cell environment could be useful in drug and protein therapy development and routine enzyme activity assays. This will allow obtaining activity values which are closer to the physiological ones rather than if tested in dilute solution. Thus, the use of synthetic polymers, allows avoiding the inconveniences and costs of cell cultures or animal manipulation in pre-clinical stages, as well as providing more realistic values for systems biology approaches.

Excluded volume is just one of the effects that a macromolecule can face inside the cell, but it has been shown to be relevant in a wide variety of biological phenomena, in particular when proteins are involved [3], which include macromolecule diffusion [4-6], macromolecular interactions [7-8], protein stability [9], conformational equilibria [10] or enzyme kinetics [10-21].

In the past years, research focus has been set on enzyme kinetics. A decent number of enzymatic reactions have been studied in crowding conditions, but still few trends are understood. In terms of Michaelis-Menten kinetic parameters, in the majority of cases maximum velocity, v_{\max} , decreases [13,14, 17-19, 21], but in a few cases the overall enzyme activity has been found to increase [11, 14-16], and the Michaelis constant, K_m , that represents the affinity of the enzyme to bind the substrate, can increase [11, 16, 17], decrease [13-15, 18, 19, 21] or remain constant [19].

One trend that has seen some light in the last years is the enzyme/obstacle size ratio. Results suggest that small enzymes reaction rates are influenced by the amount of excluded volume – that is obstacle concentration – and not by obstacle size; while bigger enzymes are affected by both obstacle size and concentration [12, 19-21].

Besides, the effects of macromolecular crowding seem to differ between diffusion-controlled reactions and activation-controlled reactions. In fact, such effect is rather intuitive

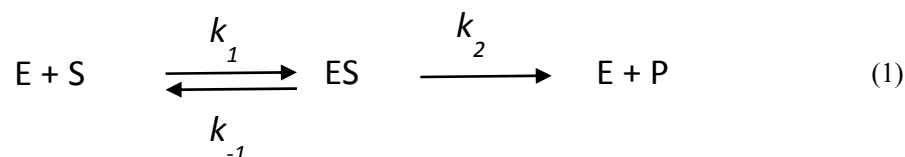
since it has been proven that macromolecular crowding can alter protein diffusion [4-6] and it can also modify conformational dynamics of the active site [22].

Both issues will be addressed in the present review, which aims to contribute in setting general trends about mechanisms by which excluded volume effects may alter the function of enzymes.

2. METHODS/MODELS

2.1. Theoretical Model

Kinetic behaviour of enzymes under crowded media conditions may be studied using the reaction scheme proposed by Henry in 1902 of a single-substrate, single-enzyme-catalysed reaction, and known as irreversible Michaelis-Menten scheme [23]:



which can be solved approximately using the stationary state assumption, SSA ($d[ES]/dt \approx 0$), which is less restrictive than the reactant stationary assumption, RSA ($[S] \approx [S]_0$), (see the recent review of Schnell for a detailed discussion) [24], to yield the well-known Michaelis-Menten equation:

$$v_0 = \frac{v_{\max}[S]_0}{K_m + [S]_0} \quad (2)$$

where v_0 is the initial velocity, $v_{\max} = k_2[E]_t$ is the maximum velocity and $K_m \equiv (k_2 + k_{-1})/k_1$ is the Michaelis constant. This assumption holds when $[E]_0 \ll K_m + [S]_0$ and it can be seen that the reactant stationary assumption, RSA, holds when $[E]_0 \approx [S]_0$ as long as $[E]_0 \ll K_m$.

Therefore, the reactant stationary assumption, RSA, is a stronger condition than the required for the steady-state assumption, SSA, and it can be seen as a necessary condition for the steady-state assumption [24].

In fact, the Michaelis-Menten equation (2) often fits the behaviour of enzymatic reactions with a known different mechanism than the one depicted in scheme (1), even for bi-substrate reactions in pseudo-first order conditions. Such easy fitting allows us to use it to approach a wide variety of enzymatic reactions, taking the values of the kinetic parameters as apparent values, allow us to generalize the Michaelis-Menten equation (2) as:

$$v_0 = \frac{v_{\max}^{app} [S]_0}{K_m^{app} + [S]_0} \quad (3)$$

where v_{\max}^{app} and K_m^{app} are the apparent maximum velocity and apparent Michaelis constant which can be put in terms of the kinetic parameters of the detailed mechanism involved [23].

In principle, in order to evaluate the effect that crowding may exert to different reaction mechanisms, numerical integration of temporal progression of the different reaction components would be necessary. This issue will be addressed in future steps. However, to evaluate the effect of macromolecular crowding in a given enzymatic reaction, obtaining apparent kinetic parameters and being able to study their fluctuations upon different experimental conditions is significant enough by itself.

2.2. Experimental Methods

Three enzymatic systems were studied in comparable conditions: bovine pancreas alpha-chymotrypsin (E.C. 3.4.21.1), horseradish peroxidase (HRP, E.C. 1.11.1.7) and rabbit muscle L-lactate dehydrogenase (LDH, E.C. 1.1.1.27), used without further purification. The three enzymes as well as all the reagents necessary for the reactions they catalyse – detailed in Table 1 - were purchased from Sigma-Aldrich Chemical (Milwaukee, WI, USA).

Dextrans, with a range of molecular weights from 5 to 410 kDa, were used as crowding agents: D5 (5 kDa), D50 (50 kDa), D150 (150 kDa), D275 (275 kDa) and D410 (410 kDa) were obtained from Fluka (Buchs, Switzerland).

Activity measurements of each enzyme were followed spectroscopically using UV-1603, and UV-1700 Shimadzu spectrophotometers through the absorption of reagents or products at the wavelengths specified in Table 1. All the experimental conditions tested during these studies are shown in Table 1 – regarding enzyme and substrate concentrations – and in Table 2 – regarding crowding agent sizes and concentrations. It is worth mentioning that since crowding agent concentrations are calculated in weight, they are directly related to the amount of excluded volume.

Moreover, all the studied systems present a negligible volume change in the reaction process (that is substrates and products are similar in size and much smaller than the enzymes).

Comprehensive experimental details and complete information about the aforementioned enzymatic reactions are described in previous references, as well as the complete results obtained for each reaction in crowded media [17-19].

Table 1: Enzymes and substrates concentrations, buffer and ionic strength experimental conditions and reaction tracking method.

Enzymes	Substrates		Buffer solution	Reaction tracking
Alpha-chymotrypsin from bovine pancreas type II	N-succinyl-L-phenyl-Ala-p-nitroanilide (0 - 4.8 · 10 ⁻⁴ M)		Tris-HCl 0.1 M pH = 8.0 10 mM CaCl ₂	Monitored by UV-vis spectroscopy at λ =410 nm (25 °C)
Peroxidase from horseradish	ABTS diammonium salt (0 - 23 · 10 ⁻⁴ M)	H ₂ O ₂ (33% aq.) (10 · 10 ⁻⁴ M)	Phosphate buffer 0.1 M pH = 7.4	Monitored by UV-vis spectroscopy at λ =414 nm (25 °C)
L-Lactate dehydrogenase from rabbit muscle	Sodium pyruvate (0 - 5.4 · 10 ⁻⁴ M)	β-NADH (1.17 · 10 ⁻⁴ M)	Imidazole-CH ₃ COOH 30 mM pH = 7.5 60 mM CH ₃ COOK 30 mM MgCl ₂	Monitored by UV-vis spectroscopy at λ =320 nm (25 °C)

Table 2: Experimental conditions assayed in crowded media: dextran molecular weight, gyration radius and dextran concentrations used.

	D50	D150	D275	D410
Dextran molecular weight, M _w (kDa)	48.6	150	275	409.8
Dextran gyration radius, R _g (nm)	5.8	11.2	14.7	17
Dextran concentrations used in crowded media experiments (g/L)	25, 50, 100	25, 50, 100	25, 50, 100	25, 50, 100

Data analysis was performed assuming the validity of Michaelis-Menten theory and thus of the steady-state approximation, which is realistic in our experimental conditions and according to previous references [17-19]. Initial velocity values (v_0) were obtained by linear fitting of the initial part of each absorbance-time plot for each single experiment mentioned in Tables 1 and 2, repeating each one for 3 to 5 times with independent samples.

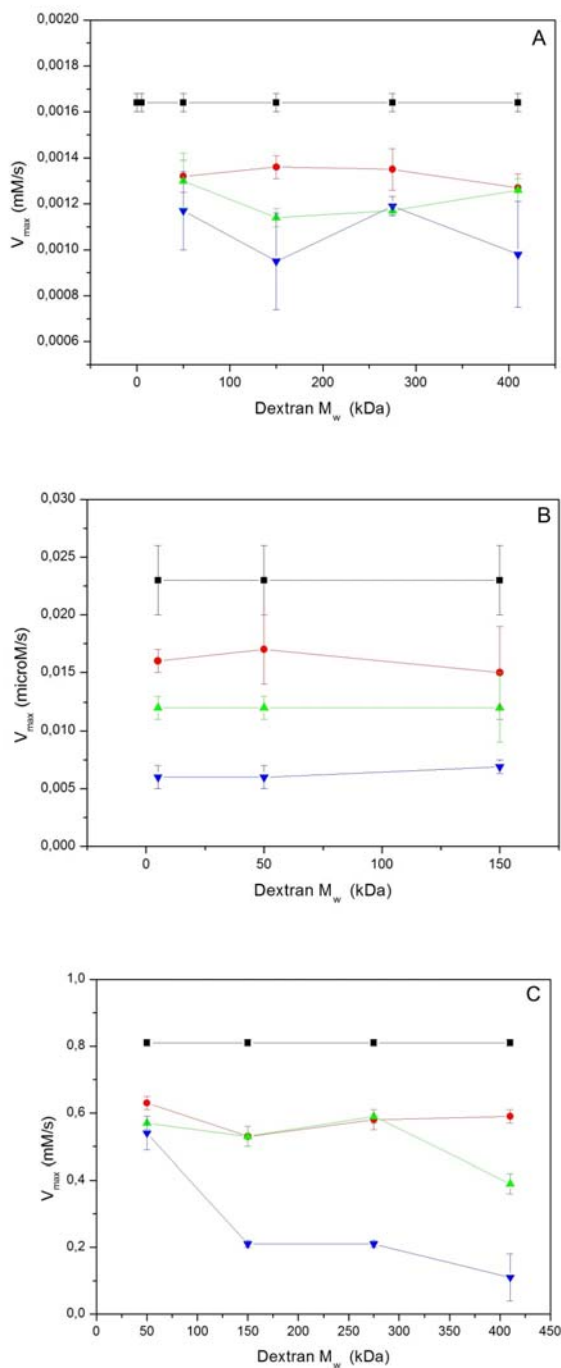
3. RESULTS AND DISCUSSION

3.1. Results

α-Chymotrypsin: 25 kDa

An initial linear raise and a subsequent plateau in the absorbance/time plot upon N-succinyl-L-phenyl-Ala-p-nitroanilide depletion were observed. Following the kinetics of this reaction under all the conditions depicted combining Table 1 and Table 2, one can observe that kinetic parameters of this reaction depend on obstacle concentration – that is excluded volume – but not on obstacle size, as seen in Figure 1A.

Figure 1: Maximum velocity (v_{\max}) versus crowding agent size (from 5 to 410 kDa) for three different enzymes: A) α -Chymotrypsin, B) HRP, C) LDH. Each point corresponds to an average value with standard deviation of 3 to 5 single experiments in different conditions: in dilute solution (black squares) and at increasing concentrations of dextran as crowding agents: 25 g/L (red circles), 50 g/L (green up-triangles) and 100 g/L (blue down-triangles).



In accordance to these results, a previous study on the diffusion of this enzyme revealed that its diffusion depended strongly on crowding agent concentration and only slightly on crowding agent size, in the same buffer and ionic strength conditions [5].

In particular, it was found that v_{\max} decreased, whereas K_m increased when increasing Dextran concentration present in the sample [17], as depicted in Fig. 2A and Fig. 3A.

Figure 2: Relative v_{\max} in dextran media for three different enzymes: A) α -chymotrypsin, B) HRP, C) LDH, in dextran concentrations ranging from 25 to 100 g/L (increasing from dark to light tone) of increasing dextran sizes: D50, D150, D275 and D410.

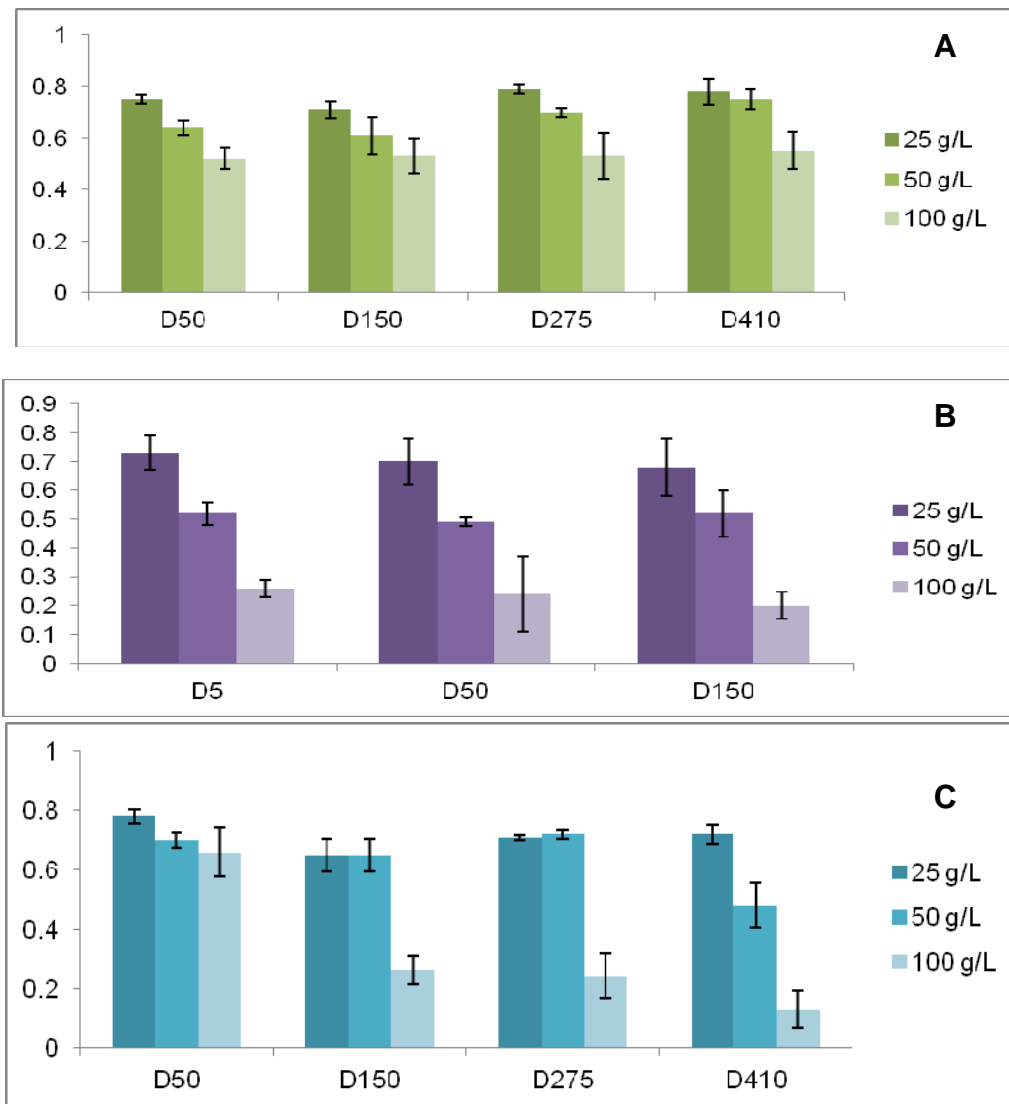
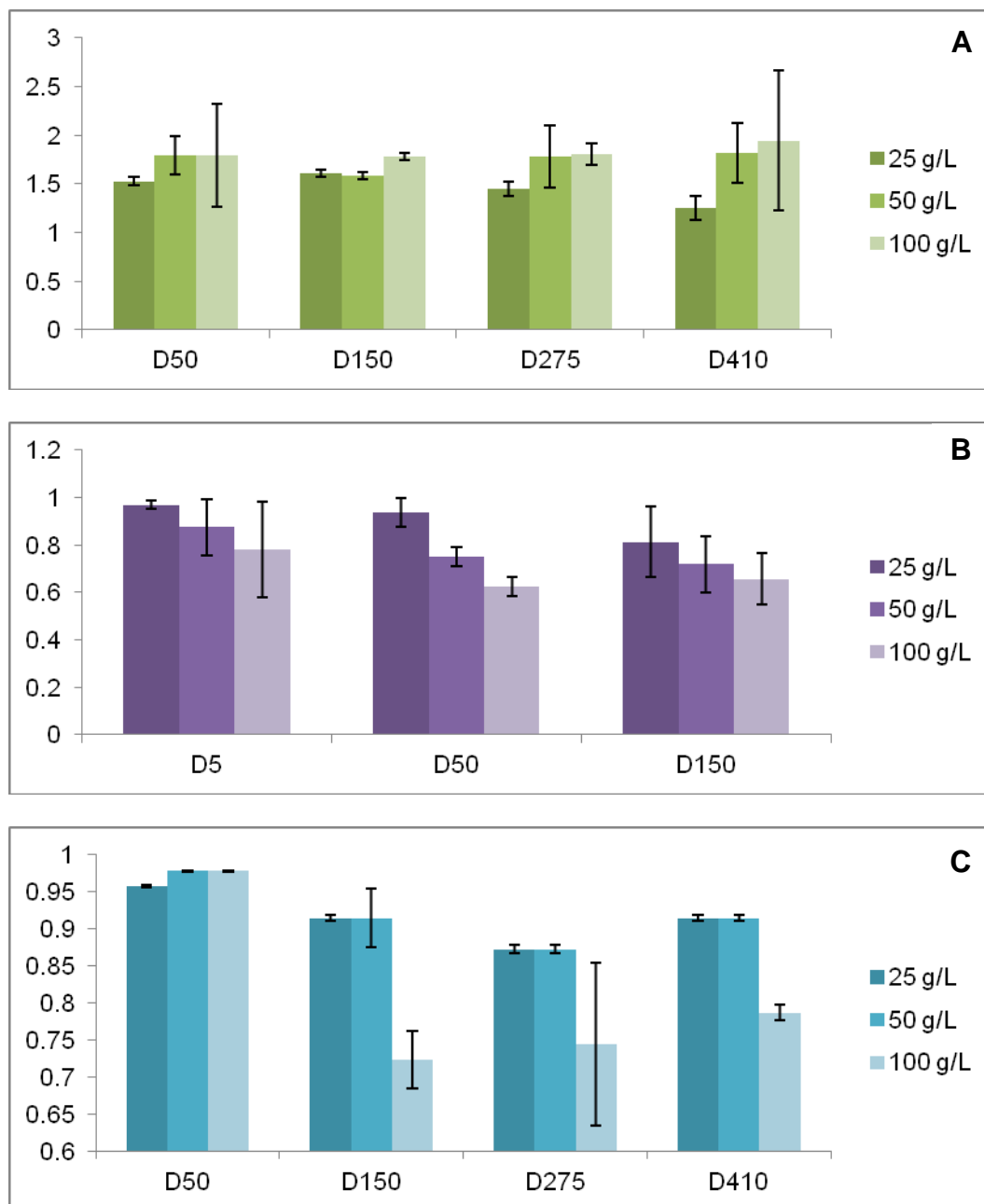


Figure 3: Relative K_m in dextran media for three different enzymes: A) α -chymotrypsin, B) HRP, C) LDH, in dextran concentrations ranging from 25 to 100 g/L (increasing from dark to light tone) of increasing dextran sizes: D50, D150, D275 and D410. Note that in figure 3C, relative K_M axis is shown from 0.6 to 1.



Horseradish peroxidase (HRP): 42 kDa

We studied the effect of macromolecular crowding in the oxidation of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) by H₂O₂ catalysed by HRP [18]. With this purpose we used this system under different concentrations and sizes of the crowding agent, as seen in Fig. 1B.

The results show that the total excluded volume by the Dextran brings a greater impact on the velocity of the reaction than the size of the crowding agent. Moreover, the results indicate that both the value of v_{\max} and K_m decay as increasing the Dextran concentration in the sample, as depicted in Fig. 2B and Fig. 3B.

In fact, this enzymatic system does not show any significant tendency when increasing the molecular weight of the crowding agent. So, regarding the obstacle size-independence, this kinetic behaviour is also shown in the previous case, α -chymotrypsin.

Lactate dehydrogenase (LDH) from rabbit muscle: 140 kDa

The oxidation of NADH by pyruvate catalyzed by lactate dehydrogenase performed in crowded media conditions reveals that the apparent kinetic parameters, v_{\max} and K_m , are dependent on both crowding agent size and concentration, as seen in Fig. 1C.

In particular, it has been found that K_m remains unaltered for all dextrans at low concentrations (25 g/L) and, at higher dextran concentrations (50-100 g/L), it shows a slight decrease for low molecular weight dextrans and a substantial decrease for high molecular weight dextrans, as seen in Fig. 2C.

Regarding v_{\max} , it always decreases with respect to diluted solution, but the decrease is significantly larger for large dextrans at high concentrations, and partially compensated for smaller dextrans and low concentrations [19], as seen in Fig. 3C.

3.2. Discussion

A schematic summary of the evidences that one can extract by analysing the crowded media kinetics of an enzyme under the generalized Michaelis-Menten equation (3) in crowded media is detailed in Table 3. Table 4 is devoted to oligomeric proteins acting as enzymes in catalysed reactions, which could yield different behaviour than monomeric proteins [12, 19-21].

The main effect of macromolecular crowding is the excluded volume effect [1] that yields an increasing value of v_{\max} (defined as $k_2[E]_t$) due to an increase of protein effective concentration. In addition, a decrease of the effective volume for the reactants is also experimentally given (Table 3). However, there are different causes that produce changes in the kinetics parameters of the enzymatic reaction. These causes can be classified in two main groups, depending if the size of the obstacle, for the same excluded volume fraction, affects or not the kinetic parameters of the enzymatic reaction.

Table 3: Effect of macromolecular crowding on proteins

K_m	v_{max}	k_I	k_2 or $[E]_t$	v_{max}/K_m	Why?	examples Refs.
↑	↑	↓	$[E]_t$ ↑	?	<ul style="list-style-type: none"> • Diffusion control • Exclude volume effect 	[11, 16]
	↓	↓	k_2 ↓	↓	<ul style="list-style-type: none"> • Diffusion control • Conformational change or k_2 is affected by changes in the environmental surroundings • Inhibition by product 	α - Chymotrypsin [17]
↓	↑	?	$k_2, [E]_t$ ↑	?	<ul style="list-style-type: none"> • Activation control • Increase in chemical activity of E and/or S in crowded media • Exclude volume effect 	Refs. [14-15]
	↓	↑	k_2 ↓	↓	<ul style="list-style-type: none"> • Activation control • More affinity for the encounters S+E • Conformational change or k_2 is affected by changes in the environmental surroundings 	HRP [18], LDH [19] and Refs. [13-14, 21]

Table 4: Effect of macromolecular crowding on oligomeric proteins, as LDH. M_c refers to the molar mass of the obstacles and M_p refers to the molar mass of the protein [19].

Particular case	Relative size	K_m	v_{max}	k_I	k_2 or $[E]_t$	v_{max}/K_m	Why?
LDH tetramer 140 kDa	$M_c < M_p$	~	↓	↓	k_2 ↓	↓	<ul style="list-style-type: none"> • Mixed activation-diffusion control • Conformational change or k_2 is affected by changes in the environmental surroundings
	$M_c > M_p$	↓	↓	↓	k_2 ↓↓	↓	<ul style="list-style-type: none"> • Mixed activation-diffusion control • Reduction of the encounters S+E for large obstacles

Effect of crowding on the reaction control

Macromolecular crowding can affect both diffusion-controlled and activation-controlled enzymatic reactions through different mechanisms of action. If we try to dissect the overall

reaction velocities in the classical Michealis-Menten scheme (1), we can analyse the effect of crowding in individual rate constants and Michaelis-Menten parameters, v_{max} and K_m .

In diffusion-controlled reactions, the reactive step is fast and the complex formation step is diffusion-dependent because a limited and/or anomalous diffusion is translate into less frequent enzyme-substrate encounters, which in turn means a decrease in k_1 . Therefore, provided that k_2 is not modified, the Michaelis constant, K_m , should increase (Table 3).

Conversely, in activation-controlled reactions, the enzyme-mediated transformation of the substrate onto the product is the limiting step. Thus, even if the enzyme and the substrates present anomalous diffusion, it will not affect the overall kinetics of the reaction, since diffusion is much faster than the product formation. However, macromolecular crowding will play another role here: when volume exclusion is not negligible, enzyme and substrate effective concentrations are undeniably higher, since the reaction volume is lower than in dilute solution, thereby causing an increase in k_1 , due to the increase of the affinity for the substrate-enzyme encounters, and therefore a decrease in K_m (Table 3).

Regarding v_{max} , several mechanisms can affect its value: it has been reported that macromolecular crowding can affect self-association equilibrium, conformational equilibrium and induce conformational changes in enzymes [2, 3, 10, 20, 22]. Subsequently, conformational changes that affect the catalytic capability of the enzyme – via slight modifications of the active site or oxyanion holes – can modify k_2 and thus v_{max} [20].

Nevertheless, the sign of this possible k_2 alteration is not clear, since the crowding-induced conformational changes may favour or hinder the interactions between the side chains of the enzyme amino acids and the substrate. And hence, for now it is not possible to predict whether v_{max} will raise or decay in crowded media [20].

As mentioned previously, another mechanism through which v_{max} (defined as $k_2[E]_t$) may be altered is because of higher enzyme effective concentration. Thus, an increase in enzyme effective concentration should result in higher values of v_{max} in crowded media. However, in the majority of studies, v_{max} is found to decrease and, consequently, volume exclusion must also cause alterations in k_2 . This contribution must be predominant over the effective enzyme concentration effect, according to the available experimental results [13, 14, 17-19, 21].

Effect of crowding on different enzyme/obstacle size ratios

As shown in the results section, in some systems with enzymes such as LDH [19], crowded media does not only affect the kinetic behaviour as a result of the amount of excluded volume, but also when increasing the size of the crowding agent.

This behaviour has been only reported for relatively big enzymes, being the malate dehydrogenase the smallest enzyme (MDH, 70 kDa) [21]. Moreover, not only enzyme size may be important in order to present this effect, but also the relative size between the enzyme and the obstacle. Existing data still lacks convergence in this matter: while some results such

as ALKP [12] and MDH [21] show that kinetic parameters are most largely affected by obstacles of a similar size as the enzyme, other enzymes such as LDH show that the largest effect occurs when obstacles are bigger than the enzyme at large amounts of excluded volume.

This size-dependence suggests that depletion forces may gain importance inside the cell cytosol, a medium in which large amount of particles of different sizes heterogeneously distributed is present.

4. CONCLUSIONS

Different consequences of high volume occupancy on the field of enzyme kinetics have been addressed: on the one hand, the obstacle size-dependent functioning of oligomeric enzymes and, on the other hand, the effect of volume exclusion upon the reaction control of enzyme-catalysed reactions.

In the first one, small enzymes such as α -chymotrypsin or HRP show an obstacle size-independent relationship, unlike bigger oligomeric enzymes such as ALKP, MDH or LDH.

The later of these, LDH, also shows an interesting behaviour when increasing excluded volume and obstacle size: v_{max} decays slightly and K_m remains constant with small obstacles at moderate concentrations, while both parameters clearly decay with big obstacles at high concentrations. These results may only be explained if the reaction control is considered as being mixed, and provided that it shifts from reaction to diffusion as crowding levels are increased.

Both findings, obtained using synthetic polymers to model volume exclusion levels typically found in the cells, remark the necessity of reconsidering traditional in-vitro enzymology and setting new bases in more biophysically realistic environments.

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REFERENCES

1. Zimmerman, S.B.; Trach, S.O. Estimation of macromolecule concentrations and excluded volume effects for the cytoplasm of *Escherichia coli*. *Journal of Molecular Biology* **1991**, *222*, 599 – 620.
2. Zhou, H.X.; Rivas, G.; Minton, A. P. Macromolecular crowding and confinement: biochemical, biophysical, and potential physiological consequences. *Annual Review of Biophysics* **2008**, *37*, 375–397.
3. Kuznetsova, I.M.; Turoverov, K.K.; Uversky, V.N. What Macromolecular Crowding Can Do to a Protein. *International Journal of Molecular Sciences* **2014**, *15*, 23090-23140.
4. Dix, J.; Verkman, A.S. Crowding effects on diffusion in solutions and cells. *Annual Review of Biophysics* **2008**, *37*, 247–263.
5. Pastor, I.; Vilaseca, E.; Madurga, S.; Garcés, J.L.; Cascante, M.; Mas, F. Diffusion of alpha-chymotrypsin in solution-crowded media. A fluorescence recovery after photobleaching study. *The Journal of Physical Chemistry B* **2010**, *114*, 4028–4034.
6. Vilaseca, E.; Isvoran, A.; Madurga, S.; Pastor, I.; Garcés, J.L.; Mas, F. New insights into diffusion in 3D crowded media by Monte Carlo simulations: effect of size, mobility and spatial distribution of obstacles. *Physical Chemistry Chemical Physics* **2011**, *13*(16), 7396–7407.
7. Zimmerman S.B. Macromolecular crowding effects on macromolecular interactions: some implications for genome structure and function. *Biochimica et Biophysica Acta* **1993**, *1216*(2), 175-185.
8. Schreiber, G.; Haran, G.; Zhou, H.X. Fundamental aspects of protein-protein association kinetics. *Chemical Reviews* **2009**, *109*(3), 839–60.
9. Wang, Y.; Sarkar, M.; Smith, A. E.; Krois, A. S.; Pielak, G. J. Macromolecular crowding and protein stability. *Journal of the American Chemical Society* **2012**, *134*, 16614–16618.
10. Minton, A.P.; Wilf, J. Effect of macromolecular crowding upon the structure and function of an enzyme: glyceraldehyde-3-phosphate dehydrogenase. *Biochemistry* **1981**, *20*(17), 4821–4826.
11. Wenner, J.R.; Bloomfield, V.A. Crowding effects on EcoRV kinetics and binding. *Biophysical Journal* **1999**, *77*(6), 3234–3241.
12. Homchaudhuri, L.; Sarma, N.; Swaminathan, R. Effect of Crowding by Dextrans and Ficolls on the Rate of Alkaline Phosphatase-Catalyzed Hydrolysis: A Size-Dependent Investigation. *Biopolymers* **2006**, *83*, 477–486.
13. Olsen, S. N.; Ramløv, H.; Westh, P. Effects of Osmolytes on Hexokinase Kinetics Combined with Macromolecular Crowding Test of the Osmolyte Compatibility Hypothesis Towards Crowded Systems. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* **2007**, *148*, 339–345.
14. Jiang, M.; Guo, Z. Effects of macromolecular crowding on the intrinsic catalytic efficiency and structure of enterobactin-specific isochorismate synthase. *Journal of the American Chemical Society* **2007**, *129*(4), 730–731.
15. Morán-Zorzano, M. T.; Viale, A. M.; Muñoz, F. J.; Alonso-Casajús, N.; Eydallín, G. G.; Zugasti, B.; Baroja-Fernández, E; Pozueta-Romero, J. *Escherichia coli* AspP activity is enhanced by macromolecular crowding and by both glucose-1,6-bisphosphate and nucleotide-sugars. *FEBS Letters* **2007**, *581*(5), 1035–1040.

16. Pozdnyakova, I.; Wittung-Stafshede, P. Non-linear effects of macromolecular crowding on enzymatic activity of multi-copper oxidase. *Biochimica et Biophysica Acta - Proteins and Proteomics* **2010**, *1804*(4), 740–744.
17. Pastor, I.; Vilaseca, E.; Madurga, S.; Garcés, J.L.; Cascante, M.; Mas, F.. Effect of crowding by dextrans on the hydrolysis of N-Succinyl-L-phenyl-Ala-p-nitroanilide catalyzed by α -chymotrypsin. *The Journal of Physical Chemistry. B* **2011**, *115*(5), 1115–21.
18. Pitulice, L.; Pastor, I.; Vilaseca, E.; Madurga, S.; Isvoran, A.; Cascante, M.; Mas, F. Influence of Macromolecular Crowding on the Oxidation of ABTS by Hydrogen Peroxide Catalyzed by HRP. *Journal of Biocatalysis & Biotransformation* **2013**, *2*, 1-5.
19. Balcells, C.; Pastor, I.; Vilaseca, E.; Madurga, S.; Cascante, M.; Mas, F. Macromolecular crowding effect upon in vitro enzyme kinetics: mixed activation-diffusion control of the oxidation of NADH by pyruvate catalyzed by lactate dehydrogenase . *The Journal of Physical Chemistry. B* **2014**, *118*, 4062–8.
20. Pastor, I.; Pitulice, L.; Balcells, C.; Vilaseca, E.; Madurga, S.; Isvoran, A.; Cascante, M.; Mas, F. Effect of crowding by Dextrans in enzymatic reactions. *Biophysical Chemistry* **2014**, *185*, 8-13.
21. Poggi, C. G.; Slade, K. M. Macromolecular Crowding and the Steady-State Kinetics of Malate Dehydrogenase. *Biochemistry* **2015**, *54*, 260–267.
22. Bismuto, E.; Irace, G. The effect of molecular confinement on the conformational dynamics of the native and partly folded state of apomyoglobin. *FEBS Letters* **2001**, *509*, 476–480.
23. Cornish-Bowden, A. *Fundamentals of Enzyme Kinetics*, 4th Ed. Wiley-VCH, Weinheim, Germany, **2012**.
24. Schnell, S. Validity of the Michaelis-Menten equation – steady-state or reactant stationary assumption: that is the question. *FEBS Journal* **2014**, *281*, 464-472.